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13. ABSTRACT (Maximum 200 Words)

Purpose: The central tenet of this proposal is that methods to effectively trigger apoptosis within prostate tumors can both reduce tumor burden and elicit adaptive immunity, provided a pro-inflammatory environment can be created.

Scope: Previously created inducible caspases (iCaspases) have been used as the basis of both a prophylactic vaccine and as treatment of pre-existing subcutaneous (sc) and autochthonous TRAMP-derived prostate tumors. While these studies are centered on prostate cancer, they could be extended to other tumor types.

Major findings: The combination of iCaspases and IL-12 can completely eliminate small ($\leq$ 40 mm^3) sc tumors and largely eliminate larger ($\leq$ 100 mm^3) tumors while IL-12 alone had minimal effect and iCaspase alone had no significant effect. Anti-tumor efficacy mirrored expansion of anti-tumor cytotoxic T lymphocytes and IFN-γ-producing cells from splenocytes of vaccinated animals. Further, orthotopic injections into the prostates of tumor-bearing TRAMP mice trigger apoptosis, secondary necrosis and inflammation, and significantly extend survival. Finally, in transgenic animals, the hK2-E3/P, PSA-E2/P and ARR$_2$PB composite promoters are highly active in prostate epithelial cells and are largely prostate specific; however, they are somewhat attenuated as tumor progression occurs, potentially reducing efficacy in tumor cells.

Significance: This work lays the groundwork for an "off-the-shelf" injectable immunogene therapy that could treat prostate cancer as a neoadjuvant therapy or possible less mutagenic treatment for metastatic disease.

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INTRODUCTION:

Currently, there are no effective treatments for men with androgen-independent metastatic prostate cancer. Among systemic therapies, including chemotherapeutic combinations, novel biological targets linked to radiopharmaceuticals, and bone-targeting bisphosphonates, all are palliative at best and usually demonstrate high morbidity associated with their mutagenicity. In contrast, new treatment modalities that target tumor antigens or tumor vasculature may treat disseminated disease with lower side effects. Immunotherapies that require patient-tailored cell culturing or knowledge of tumor antigens would be prohibitively expensive for most men. Therefore, we set out to develop a potentially injectable immunotherapy that could be used as a stand-alone or neoadjuvant therapy. This novel approach is based on tissue-specific expression of pharmacologically activated caspases that can kill slowly dividing prostate cancer cells in the primary (or secondary) tumor in a pro-inflammatory environment. We previously described the proof-of-principal of this method and further testing of vaccination of pre-existing sc and orthotopic tumors and testing of various prostate-specific promoters. Since the last report the manuscript describing this approach has been accepted by Cancer Research and will appear in May (manuscript attached). Further, we have begun developing vectors for clinical trials that include the use of a secreted HSP70 protein to potentially replace IL-12.

BODY:

Following is a list of tasks along with a summary of progress to date:

Task 1: Test the hypotheses that iCaspases can trigger apoptosis in normal and malignant prostate epithelial cells in vivo.

We previously demonstrated that iCaspases can trigger apoptosis in syngeneic TRAMP-based tumors[1] and LNCaP xenografts[2]. To demonstrate iCaspase killing in mouse prostates, we have injected ADV/CMV-iCaspase-1[1] into the ventral prostate lobes of 20-week old TRAMP mice, which should contain primarily poorly differentiated adenocarcinoma in most animals[3]. While control virus led to no obvious increased necrosis over wild-type mice in 3/3 animals, transduction with ADV/CMV-iCaspase-1 followed by intraperitoneal CID injection 3 days later, led to widespread necrosis (viewed after 10 additional days), demonstrating that prostate cancer cells are highly sensitive to caspase-1 activation. (Shown previously). Moreover, we injected ADV/CMV-iCaspase-1 into even more malignant tumors at age 32 weeks to see if

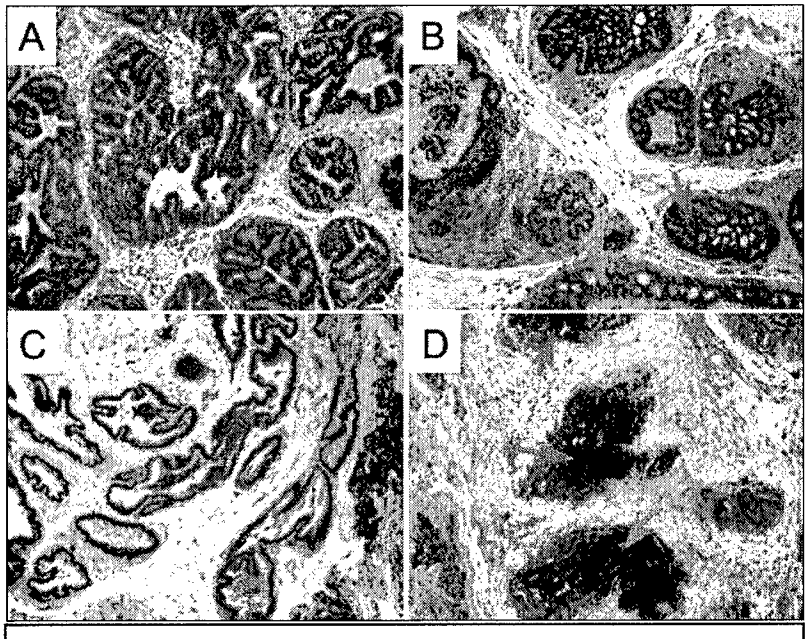

Fig. 1. Intraprostatic administration of Ad-iCasp1 followed by intraperitoneal injection of CID results in extensive cell death in prostate tissues of TRAMP mice. Ventral prostates of 20-week-old (**A, B**) or 32-week-old (**C, D**) TRAMP mice were injected once with either Ad-iCasp1 and Ad-IL-12 (**A**) or Ad-c (**C**) followed by i.p. CID carrier administration, or with either Ad-iCasp1 and Ad-IL-12 (**B**) or Ad-iCasp1 (**D**) followed by i.p. CID administration. Ventral prostate lobes were excised 20 (**A, B**) or 72 (**C, D**) hours later, and TUNEL assay was performed (counterstaining with hematoxylin). Magnification 100×. Areas with extensive cell death (brown staining) are indicated by arrows.

TRAMP tumors become resistant to Caspase-mediated apoptosis. In those experiments, widespread apoptosis occurred in prostatic ducts in a largely CID-dependent fashion with lesser, but significant apoptosis occurring in the stroma (**Fig. 1**).

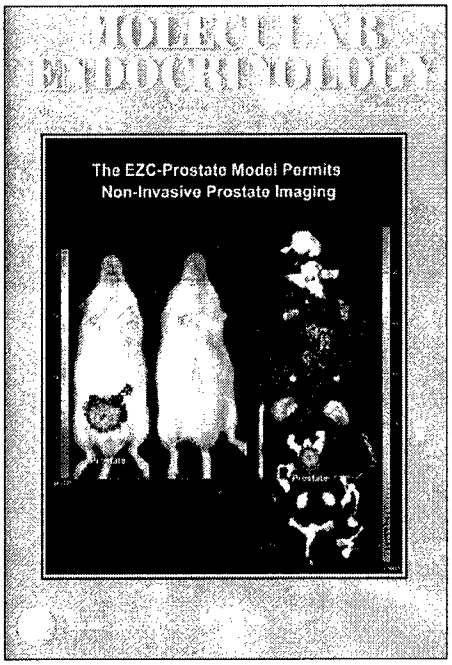

Fig. 2. Cover art from Mol Endo (3/04) showing tissue-specificity of hK2-E3/P promoter *in vivo* driving luciferase activity primarily in the prostate. Transgenic mice were injected with 1 mg D-Luciferin and imaged (for 30'') 15' later (left) with an IVIS-imaging system or ex vivo (right) ~ 45' later.

To demonstrate the utility of adenoviruses expressing tissue-restricted iCaspases, we are currently injecting adenovirus ADV/ARR_2PB-iCaspase9[2] into the ventral lobes of normal and TRAMP prostates. Moreover, we have made transgenic mice expressing luciferase under the influence of three different prostate-specific promoters, hK2-E3/P[4,5], ARR_2PB[6] and a composite PSA-based promoter, PSA-E2/P[2]. In addition to the published EZC-Prostate line based on the human kallikrein 2 promoter, hK2-E3/P **(Fig. 2)**[5], selected founder lines based on the rat probasin-based promoter, ARR_2PB, and PSA-E2/P also show high-level tissue-specific reporter expression (Fig. 3). Further, when the hK2-E3/P-Luc mice were bred onto the TRAMP background (containing prostate-targeting SV40 T antigen), we could detect metastatic prostate cancer in intact tissues using luciferase expression as a reporter **(Fig. 3).** Furthermore, when crossed with JOCK-1 mice displaying dimerizer drug inducible FGFR1 signaling and hyperplasia, the signal was maintained despite development of widespread, high grade PIN in these models. Similar experiments have been performed with the PSA and probasin-based promoters. Therefore, it is very likely that adenoviruses using the ARR_2PB promoter should express well in prostate tissue.

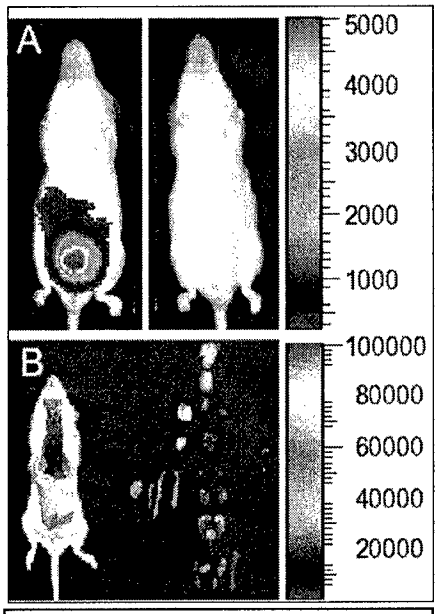

Fig 3. EZC-Prostate®: Prostate-specific luciferase expression in ARR_2PB-luc mice. A. Prostate-localized expression in 12-wk Tg mouse (left). Non-Tg littermate (right) B. Prostate-specific expression viewed *ex vivo*. **Notes:** (i) *Ex vivo* signal ~ 20 x *in vivo* due to tissue, skin, hair absorption (ii) weak ~2% signal in testes and epididymis.

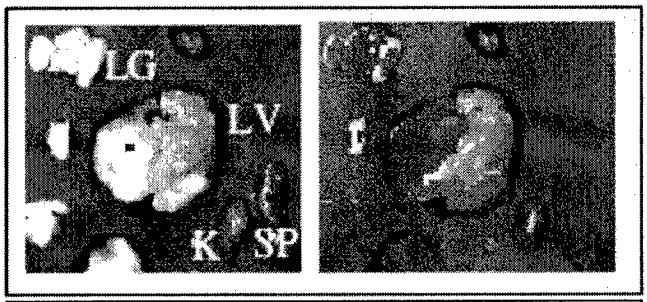

Fig. 4. Prostate Metastasis in TRAMP x hK2-E3/P-Luc mice express luciferase. 24-week bigenic mice bearing large tumor burdens were injected with 1 mg D-Luciferin. After 20', organs were dissected and arranged on black paper. 45' later, light (left) or chemiluminescent (right) microscopy was performed on Lung (LG), Liver (LV), Spleen (SP), Kidneys (K), and other tissues. Note regions of metastasis (white in left panel) correspond to luminescence, which is not seen in control mice (not shown).

Task 2,3. Test the hypotheses that inducing apoptosis in prostate adenocarcinoma cells will induce a T_H1-biased immune response. Test the hypotheses that triggering apoptosis in the context of a T_H1-induced cytokine milieu will evoke or augment an anti-tumor immune response.

Tasks 2 and 3 have been combined and are being tested simultaneously in some experiments. To test this hypothesis in sc TRAMP tumors, groups (n =5) of mice bearing small (≤ 35 mm^3) and medium ($\leq$100 mm3) tumors were injected intratumorally with adenovirus expressing iCaspase-1 (as above), ADV-IL-12 (expressing IL-12), both or neither (i.e. ADV/c), and tumor sizes were estimated (via calipers) biweekly. All groups were controlled for total viral particles. Although injection of ADV-IL12 showed some efficacy in small tumors, the combination of iCaspase-1 (+ CID) and IL-12 led to complete elimination of small tumors. No other group, including non-treated tumor-bearing mice, showed any efficacy (**Fig. 6**). Further, CTL activity and IFN-g producing cells in splenocytes from optimally vaccinated mice showed optimum expansion (**Fig. 7** and not shown). When medium-sized tumors were injected, trends were similar with optimally treated tumors averaging 10% of controls, however only 2/5 mice were completely tumor-free (not shown).

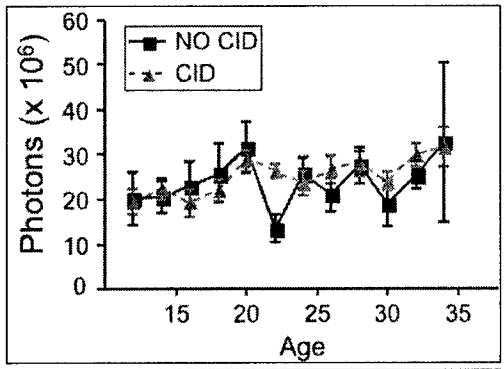

Fig 5. EZC-JOCK mice fail to reveal underlying growth in prostate development. EZC-Prostate mice were bred with JOCK1 mice and were treated with biweekly CID (2 mg/ml AP20187) or carrier alone. Total photons over urogenital region shown. Average ± Std shown (n = 3).

To test the central hypothesis of this task in autochthonous tumors, we have injected 12-week TRAMP mice intraprostaticly with ADV-iCaspase-1 plus ADV-IL-12 or control ADV/C. After two weeks a second injection was performed. Approximately 80% of mice survived the double-survival surgery. While control mice succumbed to late-stage prostate disease and were euthanized or died from high tumor burden, the Caspase/IL-12 vaccinated mice lived significantly longer (**Fig. 8**). Further, there was clear evidence of caspase-mediated apoptosis, necrosis, and infiltrating lymphocytes in caspase/IL-12 treated mice (**Fig. 9**). Similar experiments will be performed with new viruses

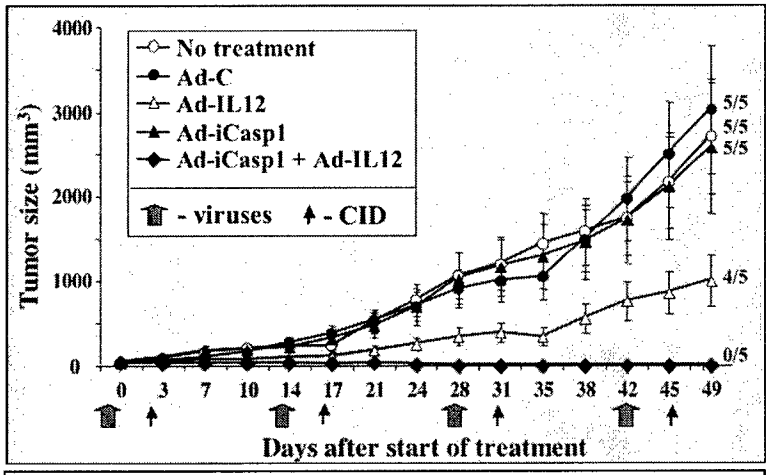

Fig. 6. Treatment of small TRAMP-C2 tumors with Ad-iCasp1 and Ad-IL-12. TRAMP-C2 tumors were established in syngeneic mice following injection of 10^6 cells. When average tumor size was ~ 35 mm^3, tumors were injected with equal numbers of adenovirus, containing IL-12, iCaspase1, both or neither (Ad-c). Control tumors were mock injected. Tumor sizes were estimated with vernier calipers. Average tumor size ± std dev. shown. Fraction of mice (out of 5) with no tumors is also shown.

expressing prostate-restricted inducible caspase-9 (i.e. ADV-ARR2PB-iCaspase9) and ADV-IL-12, as this will most likely be the virus used in a clinical trial.

Task 4. (Optional) Test the utility of using the EZC-Prostate model to measure tumor growth in vivo following vaccination. Yr 2-3.

We completed assessing the ability to monitor changes in prostate size in living tumor-prone TRAMP mice and in our inducible prostate cancer model, called JOCK, based on a dimerizer-inducible, prostate-targeted version of FGFR1. Results suggest that as the prostate becomes more hyperplastic and dysplastic luciferase activity actually decreases perhaps due to changes in promoter activity, vascularity, or both. We are currently validating Living Image® data with direct anti-luciferase immunohistochemistry. Nevertheless, enough activity remains to justify use testing of tissue-specific promoters. Since the promoters are mildly "self-limiting" in transformed tissue, we are proceeding with development of a Cre/Lox-based prostate reporter line that converts ARR_2PB-based Cre recombinase expression to a constitutive EF1α-based promoter driving luciferase. This construct will use red-shifted click beetle luciferase (from Promega) to improve diffusion through tissue.

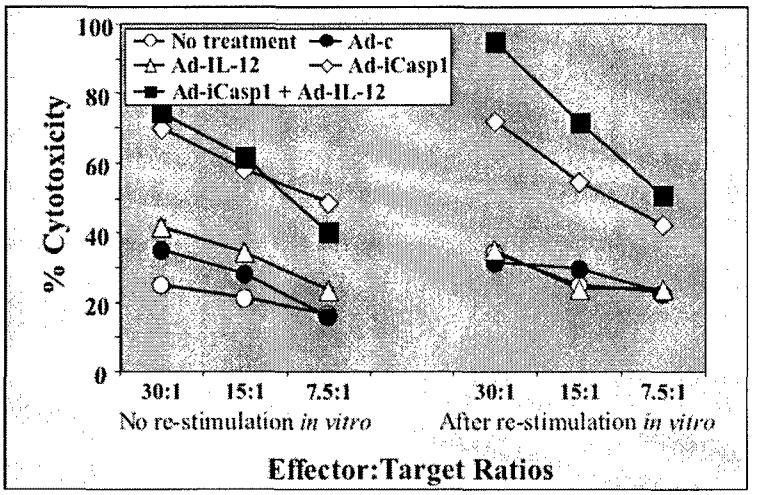

Fig. 7. Treatment of tumors with Ad-iCasp1 and Ad-IL-12 leads to potent CTL activity. Splenocytes from vaccinated mice bearing initially medium-sized tumors (≤ 100 mm^3 at first ADV injection) were cultured for either 7 d in T-Stim® without restimulation or with restimulation by TRAMP-C2-pulsed DCs. % cytotoxicity = % specific release of ^{51}Cr-loaded TRAMP-C2 target cells (pretreated with IFNγ to increase MHC class I expression). Average of 3 mice/group euthanized at conclusion of experiment (~ day 50 after first virus injection) shown.

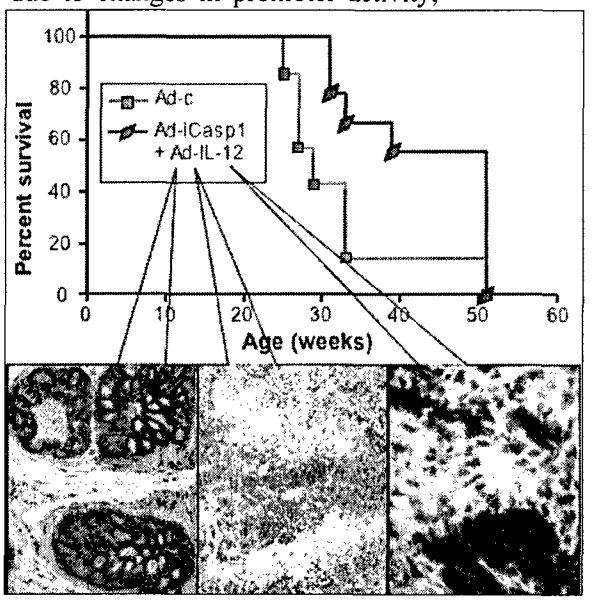

Fig. 8. Treatment of pre-existing PCa with Casp-1/IL-12 therapy. Adenovectors expressing iCaspase-1 and IL-12 or empty control vector were injected into the ventral prostate glands of 12-wk TRAMP mice. A second injection was performed on week 14. Panels: (left) TUNEL assay performed 2d after CID injection. (center) H&E stain shows necrosis. (right) stain for lymphocyte-derived acid phosphatase indicates lymphocyte infiltration.

Task 5. (Optional) Develop novel adenoviral vector, ADV-sHSP70-IRES-icp30Casp-9, coexpressing secreted HSP70 and an improved version of iCaspase-9. Year 2-3.

Independently, the Chen lab developed a related approach based on release of the potent adjuvant, HSP70, by a conditionally cytolytic virus[7]. Therefore, to compare the efficacy of IL-12, associated with systemic toxicity, with the adjuvant HSP70, we are combining in one adenovector iCaspase-based killing with their secreted HSP70.

To further improve the system, we have shifted from iCaspase-1 use to the more sensitive and less leaky iCaspase-9. Toward this goal, we have made or are making 3 separate adenoviruses. These include ADV-CMV-icp30Caspase-9 (virus finished), expressing improved Caspase-9, ADV-ARR_2PB-icp30Caspase-9 (currently testing restriction-digest verified viral plaques for protein activity and expression), based on AR-restricted expression of improved Caspase-9 and ADV-sHSP70-IRES-icp30Caspase9, expressing secreted HSP70 and iCaspase-9 (shuttle vector 50% finished). Upon completion, these vectors will be tested in both subcutaneous TRAMP-C2 and B16 tumor models and TRAMP mice as described above.

KEY RESEARCH ACCOMPLISHMENTS:

- Demonstration that iCaspases can kill prostate adenocarcinoma cells in autochthonous tumors
- Demonstration that combination vaccines with iCaspase-1 and IL-12 can eliminate small sc tumors and greatly retard medium-sized sc TRAMP tumors in syngeneic mice.
- Demonstration that combination vaccines with iCaspase-1 and IL-12 can increase survival of prostate cancer-bearing mice.
- Demonstration that all three composite prostate cell line-specific promoters, hK2-E3/P, PSA-E2/P and hK2-E3/P are active in prostate cells and prostate cancer cells in SC tumors. Although the promoters can be somewhat attenuated with progression, they are still active.

REPORTABLE OUTCOMES:

1. Ekaterina Yu. Nikitina, Smruti A. Desai, Xiuqin Zhao, Weitao Song, Annie Z. Luo, Rama D. Gangula, Kevin M. Slawin and **David M. Spencer.** (2005) Versatile prostate cancer treatment with inducible caspase and interleukin-12, Cancer Research, *in press (due May 15).*

2. *Use of iCaspase-9 suicide gene to control T cell survival in vivo. This gene was developed as part of our prostate cancer vaccine:*

Karin Straathof, Martin Pulè, Patricia Yotnda, Gianpietro Dotti, Elio Vanin, Malcolm Brenner, Helen Heslop, **David M. Spencer**, Cliona Rooney (2005) An inducible caspase 9 safety switch for cell therapy, Blood, *in press.*

3. *Paper describing new prostate reporter mice and crosses with TRAMP and JOCK is in preparation.*

CONCLUSIONS:

Our goal is to develop an injectable vaccine for advanced prostate disease that does not depend on antigen characterization or ex vivo culturing of patient tissue. Towards this goal we have demonstrated a combinatorial vaccine based on inducible caspases and the cytokine adjuvant IL-12. These are expressed in an adenoviral vector, which has been injected intratumorally. When

these vectors are injected into sc tumors, complete tumor regression is possible in small tumors and significant regression is seen in larger tumors, corresponding to expansion of tumor-specific CTL and IFNγ-expressing cells from splenocytes of vaccinated mice. We have also developed and begun testing delivery of tissue-specific iCaspases to the prostates of TRAMP mice. Initiation of intra-prostatic vaccines based on our initial vectors has shown significant efficacy, triggering apoptosis, anti-tumor immunity and prolonging longevity. During the next year, we will compare IL-12 with secreted HSP70 as adjuvants and characterize improved iCaspases, based on Caspase-9.

These studies should be the basis of a novel vaccine approach that should be easily converted to a clinical protocol provided the results show efficacy. Immunotherapy such as this is likely to be more effective and better tolerated than chemotherapies to eliminate disseminated disease, since androgen-independent metastasis often coincides with increased resistance to chemotherapy.

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7. Huang, X.F. et al. A broadly applicable, personalized heat shock protein-mediated oncolytic tumor vaccine. *Cancer Res* **63**, 7321-9 (2003).